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Comparative Study of Soluble Sulfate Reduction by Bacterial Consortia from Varied Regions of India

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Abstract: Soluble sulfate contamination in water is observed due to various industrial activities. Chemical means of reduction are available yet the biological approach is the preferred one. **Problem statement:** The problem addressed in this study was the isolation of efficient sulfate reducing bacterial consortia for bioremediation of soluble sulfate from mining effluent. **Approach:** The culture based method using the DSMZ specific media were used for isolation of sulfate reducing bacterial consortia. Their reduction efficiency was measured spectrophotometrically following growth under varied temperature and pH in specified media as well as in effluent water. The microbial consortia were analyzed at the 16SrDNA level to identify the members. The completeness as well as richness of the study was analyzed using OTU saturation curve, Shannon diversity index and equitability index. **Results:** All the eight consortia were able to tolerate vide range of pH (6-9) and temperature (20-40°C). They could reduce 63-99% of soluble sulfate (~2000 ppm) in 48 h. **Conclusion:** This study reported about the enrichment of few of the most efficient anaerobic microbial consortia that could be employed for environmental soluble sulfate reduction under diverse pH and temperature conditions.

Key words: Sulfate reducing bacteria, wastewater, bioremediation, East Calcutta Wetland, hot water spring

INTRODUCTION

Sulfate Reducing Bacteria (SRB) are a heterogeneous group of microbe, which use sulfate as terminal electron acceptor (Hansen, 1994). They use simple inorganic and organic compounds like hydrogen, ethanol, methanol, acetate, lactate, propionate and pyruvate, as electron donors (Parkes *et al*., 1989; Bak and Pfennig, 1991; Liamleam and Annachhatre, 2007) and reduce the sulfate to hydrogen sulfide as end product by dissimilatory sulfate reduction pathway (Ralf *et al*., 2006). The sulfate reducing bacteria are of wide technological interest not only for their ability to reduce soluble sulfate but also for formation of insoluble metal sulfide thus removing toxic metals from waste water (Colleran *et al*., 1995; Hammack and Edenborn, 1992; Hoa *et al*., 2007; Biswas *et al*., 2008). SRB produce high amount of hydrogen sulfide which has great affinity to react with divalent metals thus reducing them to insoluble sulfides along with reduction of sulfate in the waste water (Bai *et al*., 2008; Jimenez-Rodriguez *et al*., 2009; Neculita *et al*., 2007; Radhika *et al*., 2006; Remoudaki *et al*., 2003; Teclu *et al*., 2009; Velasco *et al*., 2008; Hsu *et al*., 2010). Thus anaerobic reduction of sulfate by SRB is the most crucial step for removal of sulfate and soluble metals from waste water (Hsu *et al*., 2010; Alvarez *et al*., 2007; Baskaran and Nemati, 2006).

Sulfate is released mainly as a byproduct of industrial activities like metal smelting, fuel gas scrubbing, molasses fermentation, tanneries, food production, coal burning power plants and pulp and paper processing (Liamleam and Annachhatre, 2007; Austin, 1984; Shin *et al*., 1997), mining. Other technological activities have resulted in the generation of large quantities of aqueous effluents that contain high levels of heavy metals (Kadukov and Vircikova, 2005).

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The techniques for sulfate decontamination are many, like reverse osmosis, distillation, ion exchange but they have many drawbacks when their efficiency is compared with the cost of implementation of the technology (Higgins *et al*., 2003). The alternative way of combating the problem is exploiting the technique of bioremediation using SRB which has been used earlier in different countries (Benedetto *et al*., 2005).

The present study deals with isolation of SRB consortia from various potential sites of Eastern India and their detailed characterization in terms of their soluble sulfate reduction efficiency, molecular identity of the organisms as well as tolerance to wide range of pH and temperature. These consortia were also tested for their sulfate reducing efficiency from environmental sample.

MATERIALS AND METHODS

Sampling site and sampling procedure: The sites selected for study were mining areas from Jharkhand (85°40'E and 22°30'N) and Orissa (21°33'18"N and 85°38'27"E), Hotwater springs at Bakreswar (87°36'E and 23°88'N) (West Bengal), Taptapani (84°40'E and 19°50'N) as well as Atri (85°30'E and 20°15'N) (Orissa) and wetland in Calcutta (88°27'E and 22°27'N), West Bengal in India. Water and soil were collected from 7 different sites at Jharkhand, 4 sites from the Orissa, 6 sites at Bakraswar, 2 sites at Taptapani, 1 site at Atri and 2 sites at East Calcutta Wetland (ECW) in India and carried in sterile plastic containers and transported to the laboratory for further analysis at ambient temperature.

Enrichment and cultivation of batch cultures: The following media according to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH (German collection of microorganisms and cell cultures) (http://www.dsmz.de/microorganisms/) specifications viz: DSMZ 63, DSMZ 163, DSMZ 16695, DSMZ 866, DSMZ 815, DSMZ 193, DSMZ 195 and DSMZ 14480 were used for isolation of enriched SRB consortia. The pH of all the media were adjusted with 5N NaOH and 6N HCl solution as prescribed by DSMZ. A 2% inoculum was used for 50ml of the medium in 100 mL serum vials under anaerobic condition as per DSMZ specification. For soil and slurry samples a dilution was made in sterile water and the particulate matter was allowed to settle down before the supernatant was used as inoculum at a similar ratio (2%). The vials were incubated at temperatures as prescribed by DSMZ for the specific medium. The cultures showing growth were analyzed for the sulfate reduction. The media were all autoclaved unless mentioned otherwise.

Media for bioaugmentation: Since the primary aim of the study was to reduce the soluble sulfate from the effluent water, bioaugmentation was tried out. For this the freshly collected effluent water was used without sterilization for the production of modified media DSMZ-16695 which was prepared by removing the sulfate containing salts from the prescribed media and replacing the distilled water with effluent water. This was done to avoid the addition of any further amount of sulfate apart from that already present in the effluent water. The sulfate concentration was measured after 12 h interval. The different consortia enriched were inoculated (2%) in separate vials of the non sterile modified media and the reduction kinetics was studied at 12 h interval for 48 h.

Sulfate measurement by turbidometric method: The sulfate measurement was done using the turbidometric technique described elsewhere (Icgen *et al*., 2006) with minor modifications. Here the sulfate ion was precipitated in hydrochloric acid medium with barium chloride to form insoluble barium sulfate crystals. The modified conditioning mixture per liter contained Glycerol (104.16 mL), concentrated Hydrochloric acid (60.25 mL) and 95% isopropyl alcohol (208.33 mL). For each reaction 2 mL of the cell free supernatant (culture harvested at 6000 g for 10 min) was taken and 1:50 dilution was made with Millipore water in a 250 mL conical flask and 5 mL of conditioning mixture was added. The entire suspension was mixed well through stirring. Under this condition approximately 1 gm of Barium chloride crystals was added and stirring was continued for exactly 1 min. The mixture was allowed to settle for 2 min under static condition before the turbidity (due to barium sulfate crystals) was measured spectrophotometrically at 420 nm. The concentration of sulfate ion was determined from the standard curve prepared using standards from 0-40 ppm of Na_2SO_4.

Physiological characterization: All the 8 consortia were separately grown at 4, 8, 20, 30, 37, 40, 50 and 60°C. The sulfate concentration was determined by turbidometric method spectrophotometrically after 24 h interval up to 96 h. Similarly they were cultivated at 5 different pH viz., 4, 6, 7, 8 and 9 and sulfate concentration was determined at 24 h interval up to 48 h.

Sulfate reduction from effluent water: Since the primary objective of the study was to look for efficient

consortia for environmental sulfate reduction, each consortium was used separately to check for its reduction efficiency in the modified media. The modification in each case was removing the sulfate containing salts from the prescribed DSMZ media and replacing the distilled water with effluent water since it contains the soluble sulfate. The rest of the conditions for growth were kept the same as that of the prescribed media. The extent of reduction was measured following 2% as well as 200% inoculation.

Molecular characterization:

DNA extraction and PCR amplification: Genomic DNA was isolated from enriched cultures of all the 8 consortia according to the protocol of Ray Chaudhuri and Thakur (2006). 16SrDNA gene fragment (1300 nt) were amplified using degenerate bacterial primers according to the protocol of Ray Chaudhuri and Thakur (2006). Briefly 5 µL of template DNA was added in PCR ready mix (Sigma). About 5 µL of 50 nM stock of each forward and reverse primers were added for both 16SrDNA as well as dsrA (Spence *et al*., 2008) gene amplification in separate reactions and the volume was adjusted to 50 µL with sterile water. The reaction conditions were 95°C for 15 min (1 cycle) followed by 95°C for 15 sec, 59°C for 30 sec, 72°C for 30 sec (40 cycles) and a final extension at 72°C for 10 min for dsrA gene while in case of 16SrDNA gene it was 92°C for 2 min (1 cycle) followed by 92°C for 1 min, 50°C for 1 min, 72°C for 2 min (40 cycles) and a final extension step of 72°C for 10 min (1 cycle).

Sequencing of 16SrDNA gene: PCR products were ligated into pTZ57R Vector according to the manufacturer's protocol (pTZ57R PCR Cloning Kit, Tauraus Scientific) and transformed into *Escherichia coli* DH5α using TSS method (Chung *et al*., 1989) of transformation. Plasmids were isolated according to modified alkali lysis method as specified by ABI-Perkin Elmer and reported elsewhere (Ray Chaudhuri and Thakur, 2006) and positive clones were screened following gel retardation assay (Ray Chaudhuri and Thakur, 2006). Positive clones were sent for partial 16SrDNA sequencing using the standard M13 primers in an ABI-automated DNA sequencer. The sequences were subjected to online BLAST analysis (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) and novel sequences were submitted to GenBank.

Saturation curve: To verify the completeness of the study for each consortium the different varieties obtained (Y axis) were plotted against the number of clones (X axis). The study was continued till much after the saturation was reached.

Statistical analysis: Shannon diversity index (H) and equitability index were calculated to understand the species evenness according to the formula (Gafan *et al*., 2005) given below:

$$H = \sum_{i=1}^{s} -(P_i * \ln P_i)$$

Where:

H = The Shannon diversity index

P_i = Fraction of the entire population made up of species i

S = Numbers of species encountered

$\sum$ = Sum from species 1 to species S

Equitability index = H/ ln(i)

RESULTS

Characterization of the consortia: Out of the 22 different combinations tried out depending on media composition and site selection, only 8 viable consortia were obtained as indicated in Table 1 showing their respective growth media.

The sulfate reduction kinetics of all the consortia were studied at 12 h interval following 2% inoculum. As evident from Table 1 (column 4), almost complete sulfate reduction (99%) occurred for the consortium 7 while for the others it varied between 63-93% (Table 1). The sulfate reduction kinetics was checked by turbidometric method. No reduction was found in case of non-sterile modified media used for bioaugmentation study without inoculation. However when the different consortia were inoculated in non sterile modified media the reduction kinetics was found to be different as in Fig. 2.

As effective reduction of sulfate was evident following addition of 2% inoculum, an attempt was made to maximize metabolism of the culture in order to understand the ultimate efficiency of reduction. 200% inoculum was added to the media with the aim to enhance the metabolic rate resulting in a cumulative efficiency of reduction. It is evident from Table 1 that sulfate reduction occurred at a higher rate for all the consortia with the consortium 7 being most efficient which could almost completely reduce sulfate concentration of ~2000 ppm with in 12 h.

All the eight consortia were found to be mesophilic; able to grow as well as reduce sulfate at the temperature range of 20-40°C with the optimum being between 37-40°C (Table 1). The consortia were also able to tolerate pH between 6-9 with the optimum being between pH 7-9 (Table 1).

Table 1: Characterization of the eight consortia

Consortia code	Site of isolation	Percentage of reduction with 2% inoculum; time (h)	Optimum temp (°C); Percentage of reduction in 48 h	Optimum pH; Percentage of reduction in 48 h	Percentage of reduction with 200% inoculum in media; time (h)	Percentage of reduction with 200% inoculum in effluent water; time (h)	Shannon diversity index	Equtability index
TBN-63	Jharkhand Mines	81.0±0.056; (48)	40; 81.0±0.015	9; 68.2±0.005	98.0±0.004; (48)	83.0±0.10; (96)	1.427	0.796
TPI-63	Jharkhand Mines	67.0±0.042; (48)	37-40; 64.0±0.009	8; 69.9±0.014	99.0±0.002; (96)	79.0±0.28; (96)	0.921	0.514
TPI-163	Jharkhand Mines	63.0±0.023; (84)	37; 30.4±0.013	7; 30.8±0.010	73.0±0.013; (84)	74.0±0.008; (96)	0.822	0.459
ETPI-163	Jharkhand Mines	71.5±0.034; (72)	37; 32.0±0.050	7-9; 38±0.020	98.0±0.024; (96)	17.0±0.020; (96)	1.027	0.573
BW-63	Bheri water, East Calcutta Wetland	69.0±0.072; (48)	37-40; 61.0±0.017	7-9; 68±0.059	70.0±0.012; (24)	51.0±0.040; (96)	1.187	0.738
RSC-163	Raw Sewage Canal, East Calcutta Wetland	67.0±0.065; (48)	37-40; 68.4±0.012	7-9; 67±0.018	74.0±0.004; (24)	66.0±0.030; (96)	1.360	0.619
BW-16695	Bheri water, East Calcutta Wetland	99.0±0.001; (36)	40; 99.0±0.001	7; 99.1±0.005	99.0±0.008; (12)	99.0±0.001; (12)	1.743	0.896
G-16695	Taptapani hot spring, Gunupur	93.0±0.833; (36)	40; 75.0±0.011	8; 88.0±0.010	99.0±0.005; (12)	99.0±0.003; (12)	0.950	0.590

±: Represents standard error, all the experiments were repeated thrice independently; the number with the strain code represents the media used for enrichment. Both media and waste water was made sterile and anoxic

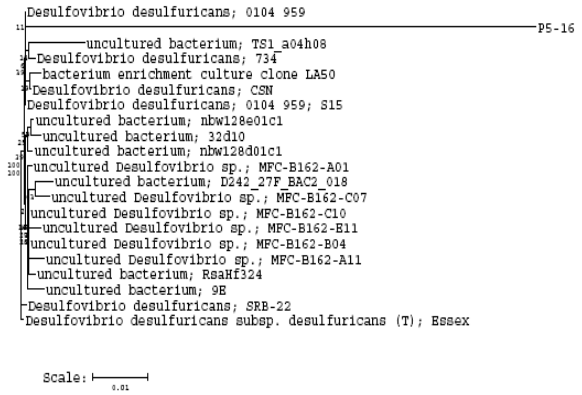

Fig. 1: Phylogenetic tree constructed using neighbor-joining method of one of the clone of consortium 7 (accession no: GQ503869) based on 16SrDNA sequence data. This clearly shows the longer distance of the isolate P16 from other *Desulfovibrio desufuricans* as evident from the long branch length

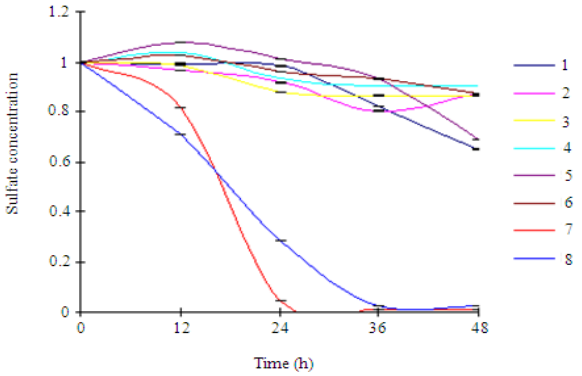

Fig. 2: Sulfate reduction kinetics of the eight consortia in modified media made with environmental effluent water (unautoclaved)

Molecular characterization: 208 clones were obtained from this screening. Apart from different groups of SRB various types of non-SRB were also found. The 208 sequences obtained had been submitted to the GenBank databases under accession numbers FJ609658-FJ609676, FJ804453-FJ804467, GQ503750-GQ503878, GQ898863-GQ898881 and GU199197-GU199222.

The phylogenetic trees were constructed using the neighbor-joining method for all the clones. A representative phylogenetic tree for one of the isolates of consortium 7 was shown in Fig. 1. Clones isolated from the hot spring were found to be more similar to thermopillic bacteria as compared to those from other sites. This was expected as they reside in high temperature. Since we did not find any known SRB species in the three consortia-TBN-63, BW-63 and G-16695 although sulfate reduction occurred, the Dissimilatory Sulfite Reductase (dsrA) gene, the key one for sulfate reduction (to catalyze the reduction of

bisulfite to sulfide), was amplified from the eight consortia confirming the presence of SRB in all the eight consortia (Fig. 3).

Saturation curve: On plotting the number of clones on X axis and the different varieties (OTU), on the Y axis, a saturation curve was obtained which clearly indicates the completeness of the study for all the consortia (Fig. 4).

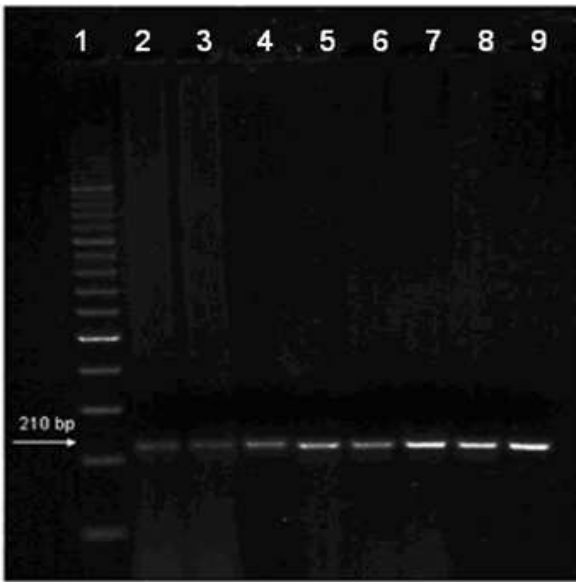

Fig. 3: Ethidium Bromide stained 2.5% agarose gel run at 100 V cm^{-2} for 4 h. The lanes were loaded with partial dsrA gene amplicons obtained from the different consortia. Lanes: 1-9 were loaded as follows: 100 bp DNA ladder (Chromous Biotech Pvt. Ltd; LAD 01), PCR amplicon from TBN-63, TPI-63, TPI-163, ETPI-163, BW-63, RSC-163, BW-16695 and G-16695 respectively

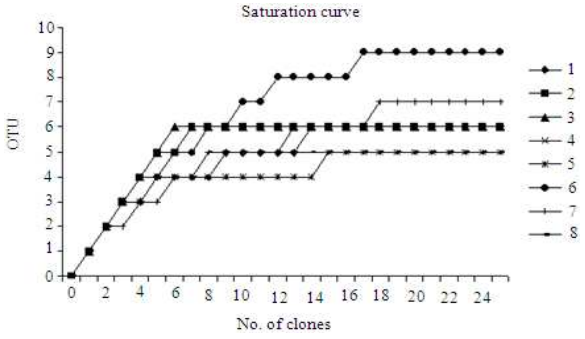

Fig. 4: The OTU saturation curve for all the eight consortia. It clearly indicates the completeness of the screening procedure in each case

Statistical analysis: High value of H (Shanon Diversity index) indicates more species diversity and richness while higher equitability index indicates species evenness. Thus Shannon diversity index and equitability index gives an indication of the diversity and nature of distribution of the species (Table 1). Statistical analysis showed that most of the population has lower value of H indicating less diversity among the population. The evenness also varies sharply among the different consortia.

DISCUSSION

We enriched eight different consortia from 22 different combinations tried out. Since the aim of the work was enrichment of SRB for its potential role in remediation of soluble sulfate from waste water, we tried to stimulate the indigenous microbiota by adding lactic acid as electron donor and the other salts required for the growth of SRB eliminating the sulfate salts. However bioaugmnentation did not work for the waste water indicating absence of efficient SRB in that site. The alternative was to obtain enriched SRB from different potential sites like wetlands and hot water springs which are expected to be rich source of SRB. All the eight microbial consortia with varied pH and temperature tolerance would have immense application in the treatment of sulfate rich effluents. All the consortia were able to survive and reduce sulfate from the effluent water indicating their potential role in waste water treatment. Increasing the percentage of inoculum to 200% (from 2%) was advantageous because it helps in faster sulfate reduction. However increasing inoculum further (400%) did not increase the reduction rate. So 200% was optimum percentage of inoculum for these consortia. The composition of the waste water as determined by Ion-Chromatography was as follows: 1286 ppm of sulfate, 2013 ppm of phosphate, 115 ppm of nitrate, 0.016 ppm of nitrite, 957 ppm of chloride and 58 ppm of fluoride.

SRB are known to use a wide variety of simple organic compounds as electron donors (Parkes *et al*., 1989; Bak and Pfennig, 1991). Electron donors that are oxidized by SRB are usually low-molecular weight organic compounds. The synthetic organic compounds reported to be used were lactate, acetate, propionate, pyruvate, butyrate, malate, ethanol, amino acids, sugars, ethanol as well as other alcohols (Parkes *et al*., 1989; Bak and Pfennig, 1991; Liamleam and Annachhatre, 2007).

Molecular characterization of the mixed consortia could give a picture of the existing population. Although all the consortia did not reveal known SRB among the 208 clones sequenced, the dsrA gene amplification clearly indicated the presence of SRB. Apart from different groups of SRB, various types of non-SRB were also found which are expected to be involved directly or indirectly with the SRB metabolic process. The predominance of non-SRB points towards the non-specificity of the selected media. Statistical analysis showed that most of the population has lower value of H indicating less diversity among the population. This is most likely due to the selective enrichment obtained on the specific medium. It is evident from Table 1 that the evenness also varies among the eight consortia. Thus along with the efficiency of reduction, the population distribution among community also varies.

CONCLUSION

This study reports for the first time the isolation of SRB consortia with a potential of reducing soluble sulfate from 2000 ppm within 36 h of incubation under optimum growth condition. The earlier reports of efficient reduction were from 2500-357 ppm in 7 days (Jong and Parry, 2003). It could function similarly for sulfate reduction from both effluent as well as medium at the laboratory scale.

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REFERENCES

Alvarez, M.T., C. Crespo and B. Mattiasson, 2007. Precipitation of Zn (II), Cu (II) and Pb (II) at bench-scale using biogenic hydrogen sulfide from the utilization of volatile fatty acids. Chemosphere, 66: 1677-1683. DOI: 10.1016/j.chemosphere.2006.07.065

Austin, G.T., 1984. Shreve's Chemical Process Industries. 5th Edn., McGraw Hill, pp: 639.

Bai, H.J., Z.M. Zhang, G.E .Yang and B.Z. Li, 2008. Bioremediation of cadmium by growing rhodobacter sphaeroides: Kinetic characteristic and mechanism studies. Bioresour. Technol., 99: 7716-7722. DOI: 10.1016/j.biortech.2008.01.071

Bak, F. and N.M. Pfennig, 1991. Sulfate-reducing bacteria in littoral sediment of Lake Constance. FEMS Microbiol. Lett., 85: 43-52. http://linkinghub.elsevier.com/retrieve/pii/037810979190630S

Baskaran, V. and M. Nemati, 2006. Anaerobic reduction of sulfate in immobilized cell bioreactors, using a microbial culture originated from an oil reservoir. Biochem. Eng. J., 31: 148-159. http://cat.inist.fr/?aModele=afficheNandcpsidt=18122240

Benedetto, J.S., S.K. De Almeida, H.A. Gomes, R.F. Vazoller and A.C.Q. Ladeira, 2005. Monitoring of sulfate-reducing bacteria in acid water from uranium mines. Miner. Eng., 18: 1341-1343. DOI: 10.1016/j.mineng.2005.08.012

Biswas, K.C., N.A. Woodards, H. Xu and L.L. Barton, 2008. Reduction of molybdate by sulfate-reducing bacteria. Biometals, 22: 131-139. DOI: 10.1007/s10534-008-9198-8

Chung, C.T., S.L. Neimala and R.H. Miller, 1989. One step preparation of competent E.coli transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA., 86: 2172-2175. http://www.pnas.org/content/86/7/2172.full.pdf

Colleran, E., S. Finnegan and P. Lens, 1995. Anaerobic Treatment of sulphate-containing waste streams. Antonie van Leeuwenhoek, 67: 29-46. DOI: 10.1007/BF00872194

Gafan, G.P., V.S. Lucas, G.J. Roberts, A. Petrie and M. Wilson *et al*., 2005. Statistical analyses of complex denaturing gradient gelelectrophoresis profiles. J. Clin. Microbiol., 43: 3971-3978. DOI: 10.1128/JCM.43.8.3971-3978.2005

Hammack, R.W. and H.M. Edenborn, 1992. The removal of nickel from mine waters using bacterial sulfate reduction. Applied Microbiol. Biotechnol., 37: 674-678. DOI: 10.1007/BF00240748

Hansen, T.A., 1994. Metabolism of sulfate reducing prokaryotes. Antonie Van Leeuwenhoek, 66: 165-185. DOI: 10.1007/BF00871638

Higgins, J.P., B.C. Hard and A.L. Mattes, 2003. Bioremediation of rock drainage sulfate reducing bacteria. Sudbury, 2: 1-7.

Hoa, T.T.H., W. Liamleam and A.P. Annachhatre, 2007. Lead removal through biological sulfate reduction process. Bioresour. Technol., 98: 2538-2548. DOI: 10.1016/j.biortech.2006.09.060

Hsu, H.F., Y.S. Jhuo, M. Kumar, Y.S. Ma and J.G. Lin, 2010. Simultaneous sulfate reduction and copper removal by a PVA-immobilized sulfate reducing bacterial culture. Bioresour. Technol., 101: 4354-4361. DOI: 10.1016/j.biortech.2010.01.094

Icgen, B., S. Moosa and S.T.L. Harrison, 2006. A study of relative dominance of selected anaerobic sulfate-reducing bacteria in a continuous bioreactor. Fluoresc. *In Situ* Hybridiz. Microb. Ecol., 53: 43-52. DOI: 10.1007/s00248-006-9009-0

Jimenez-Rodriguez, A.M., M.M. Duran-Barrantes, R. Borja, E. Sanchez and M.F. Colmenarejo *et al*., 2009. Heavy metals removal from acid mine drainage water using biogenic hydrogen sulphide and effluent from anaerobic treatment: Effect pH. J. Hazard. Mater., 165: 759-765. DOI: 10.1016/j.jhazmat.2008.10.053

Jong, T. and D.L. Parry, 2003. Removal of sulfate and heavy metals by sulfate reducing bacteria in short-term bench scale up flow anaerobic packed bed reactor runs. Water Res., 37: 3379-3389. http://cat.inist.fr/?aModele=afficheN&cpsidt=14951825

Kadukov, J. and E. Vircikova, 2005. Comparison of differences between copper bioaccumulation and biosorption. Environ. Int., 31: 227-232. DOI: 10.1016/j.envint.2004.09.020

Liamleam, W. and A.P. Annachhatre, 2007. Electron doners for biological sulfate reduction. Biotechnol. Adv., 25: 452-463. DOI: 10.1016/j.biotechadv.2007.05.002

Neculita, C.M., G.J. Zagury and B. Bussiere, 2007. Passive treatment of acid mine drainage in bioreactors using sulfate-reducing bacteria: Critical review and research needs. J. Environ. Qual., 36: 1-16. DOI: 10.2134/jeq2006.0066

Parkes, R.J., G.R. Gibson, I. Mueller-Harvey, W.J. Buckingham and R.A. Herbert, 1989. Determination of the substrates for sulfate-reducing bacteria within marine and estuarine sediments with different rates of sulfate reduction. J. Gen. Microbiol., 135: 175-187. DOI: 10.1099/00221287-135-1-175

Radhika, V., S .Subramanian and K.A. Natarajan., 2006. Bioremediation of zinc using desulfotomaculum nitrificans: Bioprecipitation and characterization studies. Water Res., 40: 3628-3636. DOI: 10.1016/j.watres.2006.06.013

Ralf, R., T.A. Hansen and F. Widdel, 2006. Dissimilatory sulfate and sulfur-reducing prokaryotes. Prokaryotes, 2: 659-768. DOI: 10.1007/0-387-30742-7-22

Ray Chaudhuri, S. and A.R. Thakur, 2006. Microbial genetic resource mapping of East Calcutta Wetland. Curr. Sci., 91: 212-217. http://cat.inist.fr/?aModele=afficheN&cpsidt=18268335

Remoudaki, E., A. Hatzikioseyian, P. Kousi and M. Tsezos, 2003. The mechanism of metals precipitation by biologically generated alkalinity in biofilm reactors. Water Res., 37: 3843-3854. DOI: 10.1016/S0043-1354(03)00306-3

Shin, H.S., O. Sae-Eun and L. Chae-Young, 1997. Influence of sulphur compounds and heavy metals on the mechanization of tannery wastewater. Water Sci. Technol., 35: 239-245. http://www.iwaponline.com/wst/03508/wst035080239.htm dated 18th April 2010. 4: 50 pm

Spence, C., T.R. Whitehead and M.A. Cotta, 2008. Development and comparison of SYBR Green quantitative real-time PCR assays for detection and enumeration of sulfate-reducing bacteria in stored swine manure. J. Applied Microbiol., 105: 2143-2152. Doi: 10.1111/j.1365-2672.2008.03900.x

Teclu, D., G. Tivchev, M. Laing and M. Wallis, 2009. Determination of the elemental composition of molasses and its suitability as carbon source for growth of sulphate-reducing bacteria. J. Hazard. Mater., 161: 1157-1165. DOI: 10.1016/j.jhazmat.2008.04.120

Velasco, A., M. Ramirez, T. Volke-Sepulveda, A. Gonzalez-Sanchez and S. Revah, 2008. Evaluation of feed COD/sulfate ratio as a control criterion for the biological hydrogen sulfide production and lead precipitation. J. Hazard. Mater., 151: 407-413. DOI: 10.1016/j.jhazmat.2007.06.004